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Ingestion and Adsorption of *Bacillus thuringiensis* subsp. *israelensis* by *Gammarus lacustris* in the Laboratory

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Several groups of *Gammarus lacustris* adults were exposed to solutions containing 0.5 and 5.0 mg of *Bacillus thuringiensis* subsp. *israelensis* per liter for 1- or 24-h periods by using traditional static bioassay exposure procedures. During a postexposure holding period, fecal pellets were removed and plated on tryptic soy agar to determine *B. thuringiensis* subsp. *israelensis* spore content. The experiments verified that traditional exposure procedures assure ingestion of *B. thuringiensis* subsp. *israelensis* spores and provided a mean dose estimate of 1,948 spores ingested per test animal with a 95% confidence interval ranging from 891 to 4,296 (1-h exposure, 5.0 mg/liter). It was also found that dose level is highly dependent upon both exposure duration and concentration and that relatively short exposures can result in a relatively long-term retention of spores postexposure (≥30 days). Body burden experiments established that large numbers of spores adsorb to the bodies of test animals during exposure and may in part explain the long-term retention of spores in the test system postexposure. These results imply that in field applications of microbial control agents, toxicologically unaffected but exposed organisms might transport the agent to untreated sites, expanding the effective treatment area and the number of organisms exposed.

Data on the effects and fate of chemical control agents in the laboratory and in the environment have been described in many reports published in the last 25 years. Data on the effects and fate of microbial control agents (MCA) are not as common but have been increasing in the last 10 years (9). Interest in the effects and fate of MCA in aquatic systems was stimulated by the discovery of a strain of *Bacillus thuringiensis* that showed specific toxicity to aquatic Diptera such as mosquitoes and blackflies. This variety, *B. thuringiensis* subsp. *israelensis*, has been extensively studied in the laboratory and in field applications (2, 3, 8, 10, 11, 16).

The studies show that this agent can be successfully used to control targeted animals and that few nontarget populations are directly affected (8). Although methods for assessing the direct effects of MCA on nontarget animals have already been adequately developed from those used for chemical control agents, methods to determine the fate of MCA in the environment are not well developed.

To determine the fate of an MCA in an ecosystem, one must first be able to predict the likely conditions in the environment under which the control agent can exist. In an aquatic ecosystem, a biological-microbial control agent can exist in one or more of the following three states: (i) suspended in the water column, (ii) adsorbed to a biotic or abiotic surface, and (iii) incorporated into living or dead organisms.

Frommer et al. (6) studied the distribution of *B. thuringiensis* subsp. *israelensis* in flowing water immediately after application. They reported that a peak in concentration occurred midway through the application period and that spore residuals in the water declined rapidly after the application was stopped. The fate of bacteria in streams was studied by Dutka and Kwan (5). They reported that bacteria do not form homogeneous suspensions in water but act as particulates, sinking to the sediment or being adsorbed to larger floating particles, and that some bacteria are caught in eddies, released or resuspended from the sediment, and then moved downstream.

Studies of this nature provide a good starting point but must be developed further to provide a more complete understanding of the movement of MCA in aquatic ecosystems. This is especially important in light of the refinement in genetic engineering techniques which have led to the development of new strains of microbial control agents that have greater potency, virulence, physiological tolerance, and host spectrum (9).

Consequently, *B. thuringiensis* subsp. *israelensis* was selected as a model sporeforming MCA for a series of experiments designed to help make predictions about the effects of a nontarget invertebrate population on the fate of this subspecies in an aquatic ecosystem.

To establish the efficacy of our test organism *B. thuringiensis* subsp. *israelensis*, a set of traditional bioassays was run on both target and nontarget invertebrates. Then, to verify that traditional bioassay procedures assure ingestion of spores and also to provide an estimate of dose level and time post-exposure that the bacilli were retained by test animals, a set of ingestion exposures was completed. One final experiment was performed to estimate the number of spores retained on the bodies of test animals after exposure because of the potential for reexposure that body surface contamination creates.

The experiments demonstrate that although the test bacilli had no observable toxic effect on the nontarget invertebrates studied, nontarget invertebrates may have a significant effect on the movement of *B. thuringiensis* subsp. *israelensis* within an aquatic ecosystem.

MATERIALS AND METHODS

Bioassay procedures. To establish the activity of the *B. thuringiensis* subsp. *israelensis* used for all the experiments reported in this paper, the following bioassay test procedures were employed.

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Test animals. The amphipod used in the following bioassays, *Gammarus lacustris*, was collected from the Moose Head River near Sturgeon Lake, Minn. Several collections were made during the study, and a colony was maintained in the laboratory on a diet of soaked hardwood leaves. The Hemipteran *Neoplea striola* and the phantom midge *Chaoborus* spp. were collected from a natural pond in Crow-Hassen Park Reserve near Minneapolis, Minn. These animals were transferred to the laboratory and exposed within a week of the collection date. *Daphnia magna* and the chironomid *Paratanytarsus* spp. were obtained from long-standing laboratory cultures. The target animal, *Aedes vexans*, was obtained from the Metropolitan Mosquito Control District in the Minneapolis/St. Paul area, and *Aedes* spp. were collected locally in Duluth, Minn. Tests using these animals were run within 1 week of collection.

Static test procedures. All animals were exposed for 96 h to a series of five concentrations in 50% reduction series with a high concentration between 30 and 35 mg of the formulated product per liter. All tests were conducted in 100- or 250-ml glass beakers, filled to 80% capacity with test water. Ten animals were exposed in each container. The exposures were conducted at 21 ± 1°C.

Test material. All tests were conducted with *B. thuringiensis* serotype H-14, 2,000 International Toxic Units/mg (3.0×10^7 spores/mg) obtained from Abbott Laboratories (North Chicago, Ill.; lot number 51-009-BR). The suggested application rate for blackfly and mosquito control was designed to obtain a water concentration of 0.5 to 3.0 mg of product per liter. This information was used to set the high concentration at 30 mg/liter for the experiments reported here. This is 10 times higher than the suggested application rate.

Test solution preparation. Test solutions were prepared by dispensing 30 to 35 mg of the product into 1 liter of Lake Superior water in a 1-liter volumetric flask. After a standardized mixing sequence, 500 ml was removed from the exposures and the remaining 500 ml was diluted to 1 liter and mixed. This sequence was continued until all five test solutions were prepared.

Ingestion and adsorption experiments. To investigate the potential effects of nontarget invertebrate populations on the distribution of *B. thuringiensis* subsp. *israelensis* in freshwater ecosystems, several experiments were performed.

Test animals. The invertebrate test species used in these experiments was the amphipod *G. lacustris*. An amphipod was chosen as the representative invertebrate for these tests because amphipods inhabit a wide range of aquatic habitats, move actively between the surficial sediments and the water column, can be collected locally, maintained easily in the laboratory, and have a discrete fecal pellet which simplifies quantification. The amphipods used in these tests were collected and maintained in the same manner as those used in the bioassays.

To verify that traditional bioassay procedures assure ingestion of spores by test animals, the following experiment was performed. This experiment also provided an estimate of dose level and the time postexposure that *B. thuringiensis* subsp. *israelensis* was retained by test animals.

Experiment 1: ingestion, confirmation, and quantification. Six *G. lacustris* adults were placed in a beaker containing 500 ml of 5.0-mg/liter *B. thuringiensis* subsp. *israelensis* solution made up in the same manner as the stock concentrations for the static bioassay tests. The animals were allowed to swim freely about the exposure beaker for 1 h. No food was provided during the exposure. After 1 h, the test animals were rinsed thoroughly under a lake water tap for approximately 30 s and then run through two consecutive 5-min, 500-ml sterile lake water baths in 500-ml beakers.

The animals were then placed into individual postexposure holding containers designed to keep the test animals isolated from one another and to prohibit reingestion of fecal pellets. This allowed estimation of dose variability between individuals and estimation of changes in pellet bacillus content over time.

The holding containers consisted of a 400-ml beaker filled with sterile lake water. In addition, a 150-ml glass jar which had the bottom replaced with stainless steel wire cloth (3-mm opening) was immersed in the 400-ml beaker. This immersed jar was raised off the bottom of the 400-ml beaker with 1-cm-long glass legs. A continuous supply of maple leaf bits which had been soaked in lake water for 30 to 60 days was maintained in each holding container for food throughout the holding period. When holding water became murky, 50% of the total volume was replaced with fresh lake water to ensure adequate dissolved oxygen levels.

Fecal pellets were removed and plated on tryptic soy agar when passed during the first 10 h postexposure and were plated at 24, 48, 72, 192, 288, 460, 624, and 720 h postexposure during the remainder of the test. Holding beakers were cleared of all pellets 24 h before each sampling point beyond 24 h. In the first 24 h, all pellets were plated and clearing was therefore unnecessary. Before being plated, fecal pellets were rinsed in 100 ml of sterile lake water in a beaker and then pipetted onto Whatman no. 1 qualitative filter paper (11 by 11 cm; Whatman, Inc., Clifton, N.J.) to remove excess moisture and minimize spore contamination on the pellet surface.

In pilot pellet-plating experiments, a pasteurizing step was included before drying on filter paper to eliminate any vegetative bacillus cells that might have been present or germinated in the guts of test animals. It was also assumed that this would increase plate count precision by eliminating other bacterial populations (12). However, this step was excluded in the experiments reported here because plate counts of pasteurized and unpasteurized samples were indistinguishable with respect to the number of *B. thuringiensis* subsp. *israelensis* colonies present. In addition, pasteurization made it difficult to handle pellets as discrete units because of breakdown during heating. The fact that pasteurized and unpasteurized plate counts did not differ with respect to the number of *B. thuringiensis* subsp. *israelensis* colonies indicates that spores ingested by amphipods do not germinate in the gut and that competition on growth media between these bacilli and other bacterial populations was not important.

Therefore, for the experiments reported here, the pellets were transferred directly from a rinse beaker to the filter paper and then to the surface of plates containing 15 to 20 g of hardened tryptic soy agar (Difco Laboratories, Detroit, Mich.). Pellet rinse water (100 μl) was added to the pellet sample on the plate. The pellets were then broken apart into fine particles with a flamed wire loop and smeared over the plate surface along with the water sample. At each sampling interval, a plate with 100 μl of pellet rinse water ("no pellet") was also smeared. All plates were incubated at 30 to 32°C for 24 h, and then the number of bacillus colonies present was recorded. Several other species of bacteria were commonly found in the samples; however, when counted after only 24 h of incubation, *B. thuringiensis* subsp. *israelensis* colonies were far more advanced than any of the other microbes seen during this study. This made identifica-

tion and counting relatively easy. Initial identifications were made through the use of Gram stains followed by microscopic examination and bioassays with colonial growth dispersed into lake water containing a *B. thuringiensis* subsp. *israelensis*-sensitive organism (a chironomid, *Paratanytarsus* spp.). *B. thuringiensis* subsp. *israelensis* colonies have a very characteristic appearance (large, white, cloudy, irregularly shaped colonies) and are very easy to identify with the naked eye after a few verifications with the methods mentioned above.

The no-pellet smear provided assurance that background spore counts in the pellet rinse water that was used to distribute pellet samples were insignificant or zero. This also helped to assure that counts from plated pellet samples came from spores within the pellet rather than from spores on the pellet surface and therefore provided an estimate of the number of spores ingested.

After verifying that traditional bioassay procedures did assure ingestion of *B. thuringiensis* subsp. *israelensis* spores by test animals, the exposure duration and concentration were manipulated to determine how the number of spores ingested varied across different exposure duration and concentration regimens.

Experiment 2: ingestion variation due to exposure duration and concentration. Experiment 2 was performed in exactly the same manner as experiment 1, except that 0.5-mg/liter bacillus solution was used as the exposure concentration instead of 5.0 mg/liter and the postexposure sampling time was shortened. Both a 1- and a 24-h exposure duration were studied.

To determine whether the amount of spores ingested and retained over time by the test animals is affected by their diet before or during the exposure (or both), the following experimental design was employed.

Experiment 3: ingestion variation due to exposure diet regimen. The methods for experiment 3 were identical to those used for experiment 2 (24-h exposure), except that four "before/during exposure" diet regimes were set up simultaneously with 12 test animals, 3 for each diet regimen. The four diet regimens were: (i) starved/starved: test animals were starved for 48 h before exposure and during the 24-h exposure period; (ii) starved/fed: 48-h starvation period before exposure and the normal maple leaf diet during exposure; (iii) fed/starved: normal diet before exposure, starved during exposure; and (iv) fed/fed: normal diet before and during exposure.

As an attempt to understand how spores were retained in the test system and passed in the fecal pellets of test animals over extended periods of time, a final experiment was performed.

Experiment 4: body surface adsorption estimates. Four amphipods were exposed to 0.5-mg/liter bacillus solution for 24 h in the same manner as in previous experiments (leaf food was provided during exposure). After exposure, the test animals were thoroughly rinsed under a lake water tap for approximately 30 s and then placed into 150-ml dilution bottles filled with 100 ml of sterile lake water. The animals were left to swim freely about the dilution bottles for 24 h. Leaf food was provided. After the 24-h swim, a 1:1 sample was prepared from each dilution bottle and plated in the usual manner. *B. thuringiensis* subsp. *israelensis* colonies were counted after a 24-h incubation period. Once the samples were plated, test animals were placed in another set of dilution bottles prepared exactly as before. This procedure followed by plating an undiluted sample was repeated at 48, 72, and 168 h postexposure.

TABLE 1. *B. thuringiensis* subsp. *israelensis* spore ingestion and quantification in experiment 1[a]

Day postexposure	$\bar{x}$	95% CI[b]	n[c]
1	19.7	11.4–33.8	34
2	7.6	4.7–12.1	17
3	14.3	7.5–23.6	14
4	13.2	7.3–23.6	14
8	4.6	3.5–6.1	20
12	3.7	2.8–4.9	16
19	1.5	1.2–1.9	14
26	1.7	1.3–2.4	13
30	1.6	1.3–1.9	11

[a] Means and their associated confidence intervals are the exponentials of log-transformed values and are based on spores per pellet; confidence intervals are based on the *t*-statistic.
[b] CI, Confidence interval.
[c] *n*, Number of samples.

RESULTS

The nontarget animals that were tested in the static bioassay exposures showed no effect at any of the five concentrations tested. No concentrations greater than 30 to 35 mg/liter were tested. The target animals tested, *Aedes* and *Paratanytarsus* spp., showed effects of the bacilli within hours of the beginning of the exposure. Even at the lowest concentration, all animals were killed within the 96-h exposure time.

The nature of the experimental design used in experiments 1 to 4 resulted in arithmetic mean spore counts per pellet that are positively correlated with the variance. Therefore, the mean values discussed in this paper are geometric means of $(n + 1)$. A summary of these data appears in Tables 1 to 4 along with the associated 95% confidence intervals and the number of samples per mean.

Experiment 1 verified that traditional bioassay exposure procedures assure ingestion of spores by the amphipod *G. lacustris*. The results of this experiment (Table 1) show a gradual decline in the mean number of spores per fecal pellet over time postexposure from a high of 19.5 to a low of 1.5 by 460 h. One-way analysis of variance indicates a highly significant difference in mean spore count across sampling intervals ($P < 0.001$). The 24-, 72-, and 96-h intervals were significantly higher in mean spore count than were the 192- and 288-h intervals ($P \leq 0.05$), which in turn were significantly higher than the 460-, 624-, and 720-h intervals ($P \leq 0.05$).

Another result from this experiment pertains to dose level. In this paper, dose level refers to the estimated number of *B. thuringiensis* subsp. *israelensis* spores ingested by a test animal as the result of the original exposure. An estimate of the mean dose of spores per test animal can be made by using the following formula: average dose per animal = $(\bar{X}_s \pm 95\%\ \text{CI})\bar{X}_{pi}$, where $\bar{X}_s$ represents the mean number of spores per pellet over the entire test period, CI stands for the two endpoints of a confidence interval (both $\bar{X}_s$ and the 95% CI are the exponentials obtained after analyses were performed on log-transformed data), and $\bar{X}_{pi}$ represents the total number of pellets produced during the postexposure holding period divided by the number of individual test animals. With this formula, the average spore dose per animal in experiment 1 was calculated to be 1,948 spores with a 95% confidence interval ranging from 891 to 4,296.

Overall, this experiment demonstrates that traditional bioassay exposure procedures result in spore ingestion by amphipods and that a short exposure can result in long-term

TABLE 2. *B. thuringiensis* subsp. *israelensis* spore ingestion variation due to exposure duration and concentration in experiment 2[a]

Day postexposure	1-h exposure			24-h exposure		
	$\bar{x}$	95% CI[b]	n[c]	$\bar{x}$	95% CI	n
1	1.8	1.4–2.4	25	11.5	5.4–24.5	25
2	1.5	1.2–1.9	23	5.6	3.6–8.8	25
4	1.3	1.1–1.6	24	5.9	4.0–8.7	23
8	1.9	1.6–2.3	20	3.4	2.7–4.4	24
15	1.4	1.2–1.6	20			
17				1.3	1.1–1.6	24
22	1.1	1.0–1.2	24			

[a] See Table 1, footnote *a*.
[b] CI, Confidence interval.
[c] *n*, Number of samples.

retention of *B. thuringiensis* subsp. *israelensis* by test animal postexposure.

Experiment 2 expresses the effect of exposure time on spore retention in *G. lacustris*. After 24 h of exposure at 0.5 mg/liter, the fecal pellets contained an average of 11.5 spores per pellet and contained a minimum of about 1.4 spores per pellet by 16 days postexposure. In the 1-h exposure at 0.5 mg/liter, the initial pellet count was <2 spores per pellet and did not change up to 22 days postexposure. The data from the two exposures confirm that *B. thuringiensis* subsp. *israelensis* is retained by amphipods at low concentrations for a time much longer than the exposure time and that, as expected, dose level is highly dependent upon exposure duration and concentration (Table 2).

The third experiment was designed to determine the effect of exposure conditions on spore uptake. Of the four feeding regimens tested, the highest bacillus content in fecal pellets was found in animals that were fed before and during the exposure, and the lowest number of spores was generally found in pellets from animals that were starved before and during the exposure (Table 3). However, two-way analysis of variance revealed that none of the differences in mean spores per pellet between the four feeding treatment groups were statistically significant ($P = 0.05$). This indicates that ingestion is relatively insensitive to changes in before- and during-exposure feeding conditions compared with the effects of changes in bacillus exposure duration or concentration.

Results of the final experiment are summarized in Table 4. Because body burden estimates were based on measurements of spore loss from the surface of exposed animals rather than measuring the amount of bacilli on the body surface directly, the estimates reported here are conservative. Despite its conservative nature, this experiment demonstrates that large numbers of spores are retained in the test system adsorbed to the bodies of test animals for at least several days postexposure. After 24 h, the mean concentration of *B. thuringiensis* subsp. *israelensis* spores in the dilution bottle bath water was 725 spores per ml. The number of spores decreased exponentially over the next 6 days, and by 168 hours the mean count was 12 spores per ml.

TABLE 4. Body surface adsorption of *B. thuringiensis* subsp. *israelensis* spores to *G. lacustris* in experiment 4[a]

Day postexposure	$\bar{x}$	95% CI[b]	n[c]
1	72.5	43.4–120.3	8
2	8.4	4.5–15.8	8
3	2.9	1.8–4.6	8
7	1.2	0.9–1.6	8

[a] Means and their associated confidence intervals are the exponentials of log-transformed values and are based on spores per 100 μl; confidence intervals are based on the *t*-statistic.
[b] CI, Confidence interval.
[c] *n*, Number of samples.

DISCUSSION

Because bioassays with *B. thuringiensis* subsp. *israelensis* that involve the use of conventional bioassay exposure procedures developed for chemical control agents typically do not result in any toxicity to nontarget organisms, it is important to demonstrate that organisms tested in this manner are actually being exposed. That is, it must be shown that spores have been ingested and actually enter the gut of test organisms. de Barjac (4) and Thomas and Ellar (15) have shown this to be the site of pathogen activity for *B. thuringiensis* subsp. *israelensis* (for a review, see reference 8).

The ingestion experiments performed for this study verified that although conventional bioassays (where the agent is suspended in water) assure ingestion of spores by *G. lacustris* (Table 1), the number of spores ingested was relatively low when compared with the number that target species have been found to ingest under similar exposure conditions (1, 14) and when compared with the number estimated to be adsorbed to their exoskeleton (Table 4).

Although the number of spores ingested was relatively low, the time that spores were found in the pellets was much longer than expected based on what is known about gut throughput time for *Gammarus* spp. Welton et al. (17) estimated the gut throughput time for *Gammarus pulex* to be about 1 h when the water temperature was 13.8°C. Unpublished data from our laboratory for *G. lacustris* estimated throughput time to be 6 to 8 h when the water temperature was approximately 15°C. Thus, it seems reasonable to expect that guts of test animals should be free of spores in less than 1 day postexposure, assuming no spore germination and

TABLE 3. *B. thuringiensis* subsp. *israelensis* spore ingestion variation due to "before/during exposure" diet regimen in experiment 3[a]

Day postexposure	"Before/during exposure" group											
	Starved/starved			Starved/fed			Fed/starved			Fed/fed		
	$\bar{x}$	95% CI[b]	n[c]	$\bar{x}$	95% CI	n	$\bar{x}$	95% CI	n	$\bar{x}$	95% CI	n
1	6.5	4.4–9.6	18	5.8	3.3–10.0	9	12.4	7.7–20.1	12	16.9	3.8–75.2	12
2	5.1	2.8–9.1	9	10.3	6.8–15.5	15	8.8	5.8–13.6	15	5.6	4.0–7.8	12
4	2.1	1.2–3.7	9	3.3	1.8–6.0	9	4.5	2.8–7.3	9	4.9	3.2–7.7	9
8	2.4	1.2–4.7	9	1.5	0.9–2.5	9	3.8	2.5–5.9	9	2.3	1.2–4.5	9
16	1.0	0.6–1.6	9	1.1	0.7–1.7	6	2.1	1.5–3.0	9	0.8	0.4–1.5	5

[a] See Table 1, footnote *a*.
[b] CI, Confidence interval.
[c] *n*, Number of samples.

subsequent sporulation in the test animals. Although there is some evidence that germination and resporulation may occur in certain target species (7), the pilot pasteurization work discussed earlier (see Materials and Methods, experiment 1) indicates that this is an unlikely possibility for our test species, *G. lacustris*. The low overall number of *B. thuringiensis* subsp. *israelensis* colonies observed in experiments 1 to 3 also argues against this possibility.

Spore germination and recycling in the holding containers is also of concern because of its potential impact on ingestion and dose estimates. However, all evidence from current work in our laboratory indicates no germination of spores in any aqueous environment without the presence of a high-protein, pH-basic growth medium. *B. thuringiensis* subsp. *israelensis* spores mixed in lake water, lake water plus sand, lake water plus potting soil, and lake water plus leaf litter have never been found to germinate in our laboratory based on pasteurized versus unpasteurized sample comparisons. Evidence from the literature implies that the target animal gut is necessary for germination (1) and that in the absence of the proper gut conditions spores are rapidly deactivated in both aquatic (8) and terrestrial (13) applications. Therefore, concerns about spore germination in the holding containers and its potential impact on the results reported here seem unfounded.

Based on the results of experiment 4, it seems more likely that the test animals were ingesting spores which had adsorbed to their exoskeleton. The amphipods used in these tests were observed preening themselves during a large part of the time they were active in the holding chambers. This preening activity created the potential for continual reexposure in the holding chambers for at least 1 week beyond the original exposure (Table 4). There is evidence from experiment 1 that the potential for reexposure due to body surface contamination extended even longer than 1 week. When animals died during the holding period of this test, their bodies were smeared onto growth media to check for the presence or absence of *B. thuringiensis* subsp. *israelensis* contamination on their body surfaces. The bacillus was evident at low levels for as long as 40 days postexposure on plates smeared in this manner. Hence, it seems likely that the longer than expected retention time was related to body surface adsorption by spores during exposure and preening of that surface postexposure in the holding chambers. It is also interesting that when newborn progeny were smear plated in the same manner as described above for dead adults, *B. thuringiensis* subsp. *israelensis* colony growth was seen in 7 of 8 instances. These progeny were all born during experiment 3 at least 1 week postexposure.

The most important implications of the findings from these experiments concern the effects of nontarget animal populations on the fate of MCA in natural aquatic ecosystems, especially with respect to the persistence and distribution of MCA in these systems.

The fact that an MCA can be retained for long periods of time on the body surface of species that are unaffected by a treatment indicates that estimates of persistence and treatment coverage may be inaccurate. Toxicologically unaffected but exposed organisms might transport the agent to untreated sites, expanding the effective treatment area and the number of organisms exposed. These same organisms would be creating a potential for reexposure of sympatric and adjacent populations for longer than would be expected from the initial treatment. Questions about persistence and effective treatment area seem particularly relevant in light of the current controversy regarding the experimental release of genetically engineered microorganisms into natural environments. Careful field research with naturally occurring MCA, including close monitoring of nontarget populations, could provide insight into these problems.

The general method of using fecal pellets to estimate dose level and retention time could be adapted to estimate effective treatment area in the field and seems feasible with a wide range of organisms.

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